Resisting Bacterial

Los Alamos scientists are taking an in-depth look at how bacteria defeat death-by-antibiotics.

SOME BACTERIA ARE ESPECIALLY TOUGH. For billions of years, bacteria have evolved numerous mechanisms to protect themselves from toxic chemicals in their environment—some of which we humans now use as antibiotics. Applying their time-tested methods of thwarting chemical threats, these hardy microbes are responsible for nearly two million antibiotic-resistant infections annually in the United States.

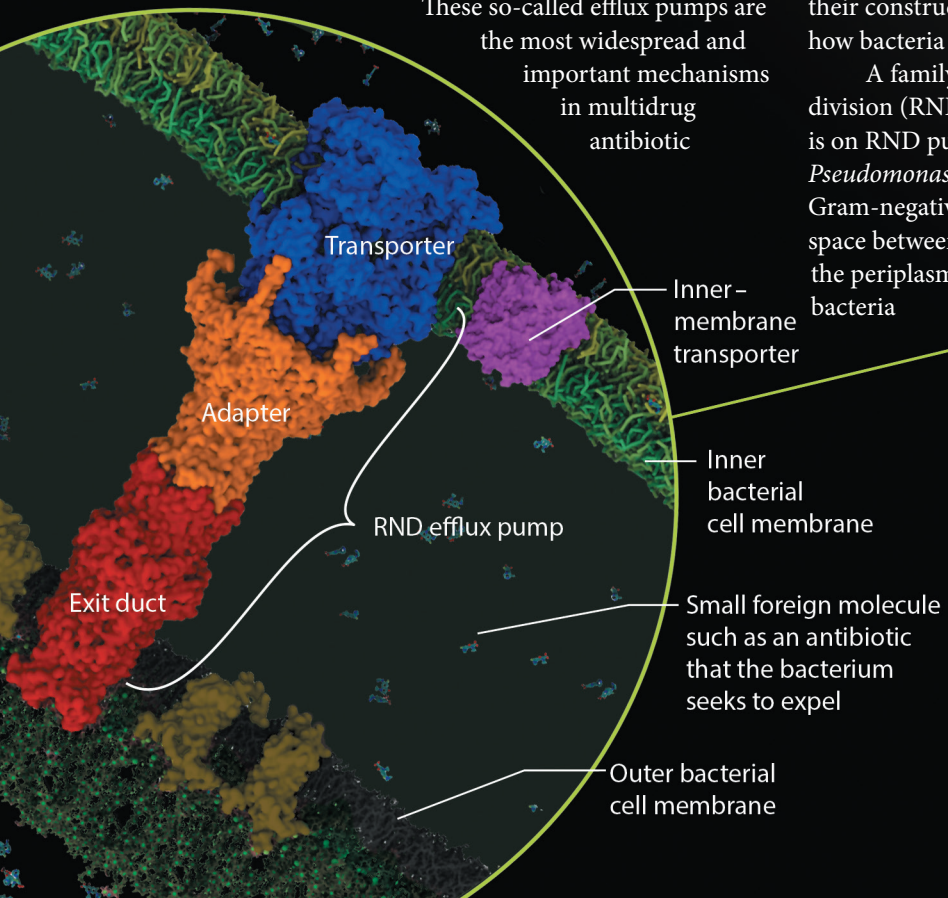
Bacteria have developed many types of defenses. Like layering for winter, some microbes wear complex coatings to serve as physical barriers to the outside world. In addition, bacteria can literally stick together to create an even stronger barrier and communication network, through which they share their defense strategies. Bacteria have even developed mechanisms to spit out any foreign toxins that get through the barriers. But if those toxins are antibiotics, intended to protect a patient against the targeted bacteria, then the patient's ability to conquer the infection might be compromised.

This spitting-out system is made possible by a set of proteins that work together as a molecular machine to pump the antibiotics out of the bacterium. These so-called efflux pumps are the most widespread and important mechanisms in multidrug antibiotic resistance. However, a complete and quantitative picture of efflux-mediated resistance is lacking. And this is a problem because bacteria are becoming resistant to more and more of the available antibiotics, putting humanity on the brink of a public-health crisis.

A multidisciplinary team of Los Alamos scientists is working on a project to understand how efflux pumps work. The researchers' goal is simple: to deactivate the bacteria's efflux pumps, thereby preventing the world's supply of antibiotics from becoming defunct. [*To learn more about the problem of antibiotic resistance, see "The Mold Rush" in the October 2015 issue of* 1663].

Gnana Gnanakaran, a theoretical biologist at Los Alamos, is the lead for the project targeting bacterial efflux pumps. Gnanakaran has gathered together a world-class collection of scientists connected by the Laboratory but widely diverse in their expertise. The group includes nearly 20 researchers, divided into teams: mathematicians and computational modelers, molecular and cell biologists, and structural biologists. Their task is to dissect all aspects of efflux pumps—their construction, how they pump out noxious molecules, and how bacteria regulate their production and use.

A family of efflux pumps called resistance-nodulation-division (RND) is the target of the team's research. The focus is on RND pumps from two types of gram-negative bacteria, *Pseudomonas aeruginosa* and *Burkholderia pseudomallei.* Gram-negative bacteria have two membranes, with a space between the membranes called the periplasm. These bacteria



Bacterial efflux pumps are molecular mechanisms for removing toxins—including antibiotics. Learning to thwart their operation by understanding how the pumps work, how they are regulated, and their role in bacterial communities is key to defeating a major type of antibiotic resistance.

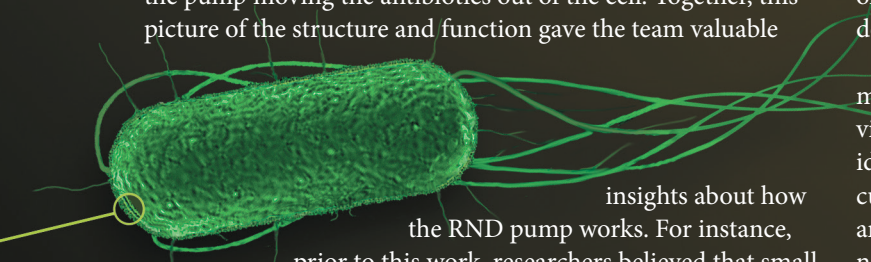
Resistance

also have many types of efflux pumps—some that span just one membrane and others, such as the RND, that span both. Together, these characteristics make gram-negative bacteria seemingly indestructible.

"We want to learn more about the assembly of these pumps and how they are utilized so that we can disable them to ensure antibiotics stay inside the bacteria," Gnanakaran says. Furthermore, a few compounds already manage to clog efflux pumps, so the researchers want to know more about those inhibitory mechanisms so more effective drugs can be designed.

Structural biologist Tom Terwilliger and his team approached understanding RND pumps by first looking at models of protein structures other researchers had made using various protein visualization techniques. RND pumps are made up of three proteins that work together to span the two membranes of the bacterium—a transporter stuck in the inner membrane, an adapter that holds the whole thing together in the periplasm, and an exit duct stuck in the outer membrane.

Using this information about the RND structure, Gnanakaran and other theorists used computer simulations to suggest how the proteins might fit into the inner and outer membranes. They identified channels through the pump where the antibiotics might enter and developed an atom-scale computer model to demonstrate the function of the pump moving the antibiotics out of the cell. Together, this picture of the structure and function gave the team valuable insights about how the RND pump works. For instance, prior to this work, researchers believed that small and large molecules entered the RND pump differently, but the Los Alamos model suggests that antibiotics of all sizes are first pushed into the periplasm by a separate type of inner-membrane transporter and then move into the RND pump to exit the cell.



"We hope to visualize this mechanism directly by determining the three-dimensional structure of RND pumps from *Burkholderia pseudomallei* with antibiotics bound to them," says Terwilliger. His team has obtained crystals of these proteins (bound with antibiotics) and plans to use x-ray diffraction to visualize the proteins in these crystals.

However, Gnanakaran's vision is to understand more than just the structure and function of the proteins; by investigating the genetic regulation of the RND pump and bacterial cells' interactions with each other, he hopes to elucidate more about the pump's overall use. Cell biologists Goutam Gupta and Kumkum Ganguly and their team carried out this portion of the project by using a knockout strain—one that does not have the gene to produce an RND pump. The knockout allows the researchers to study what happens when antibiotics accumulate within an organism that cannot make an RND pump. By sequencing the genes that the knocked-out bacteria turned on at specific points in time, the scientists inferred which proteins were being made—and found an overexpression of other types of efflux pumps.

"This is a really important observation," says Gnanakaran. "This means the bacteria can compensate for the loss of one pump by producing others."

But that's not all. Bacteria live in tight-knit communities and use specific molecules to communicate with each other. These molecules can signal the need to create a biofilm—a network of protein, DNA, and sugar that helps bacteria stick together as a barrier against intruders—by stimulating specific gene networks. When the Los Alamos team studied an overexpresser strain of bacteria, one that has a much higher number of pumps at work, it found there was also an increase in the formation of biofilms and the production of virulence factors, which are molecules that make a microbe more pathogenic. This further demonstrates that when foreign molecules, such as antibiotics, are prevalent, bacteria create more pumps to get rid of the molecules and tell their neighbors to stick together and do the same.

Now, the team has developed models to describe mathematically the interconnections among bacterial metabolism, virulence, biofilm formation, and antibiotic resistance to identify novel treatment strategies. These models go beyond the current conventional models in describing the accumulation of antibiotics inside bacteria. They illustrate how these interconnections define cellular states and can vary between pathogens.

This type of approach that examines different systems—such as how proteins work together as a machine, the genetic codes that control these machines, and the relationships between individual bacteria—is often referred to as systems biology. By evaluating these integrated models, the research team hopes to identify drugs that can simultaneously avoid multiple pumps.

With such highly evolved resistance strategies, humans face an all-out war against bacteria that will do anything they can to survive. Although there are many factors contributing to the looming antibiotic-resistance crisis—including the overuse of antibiotics and a deficit in developing new ones—a comprehensive understanding of how bacteria defy drugs in the first place is an invaluable piece of the puzzle. LDRD

—Rebecca McDonald